



REGULAR ARTICLE

# Induction of spontaneous neo-angiogenesis and tube formation in human endometrial stem cells by bioglass



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## KEYWORDS

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Bioglass

**Abstract** Endothelial dysfunction is a broad pathological disorder of the endothelium (innermost layer of blood vessels) which is assigned by vasoconstriction, thrombosis and ischemic diseases, alone or with other disorders such as coronary artery disease, hypertension and atherosclerosis. The fundamental imperfection of endothelial layer injury due to decrease in the number of functional endothelial progenitor cells and inhibition of endothelial progenitor cell differentiation, resulting into impairment of angiogenesis, vasculogenesis, tube formation properties and endothelial regeneration. Multiple significant therapeutic achievements in impediment and treatment of vascular diseases include the use of antithrombotic agents, statin class of drugs, lifestyle changes, and revascularization therapies. Nevertheless, a certain number of patients with endothelial dysfunction disease are resistant to the usual therapies, so new therapeutic strategies for endothelial dysfunction disease are urgently needed. Recent studies show that stem cell-based therapy has important promise for repair and treatment of vascular dysfunction. In this study, we describe a novel choice for treatment of endothelial dysfunction in vascular regenerative medicine via the human endometrial stem cell culture (as a new source for the increasing the number of endothelial progenitor cells) with bioglass (angiogenic agent) to investigate the enhancing expression of CD34, CD31 and gene markers of endothelial progenitor cells and endothelial cells. In the end, application of immunoprivileged, readily available sources of adult stem cells like human endometrial stem cells with bioglass would be a promising strategy to increase the number of endothelial progenitor cells and promote spontaneous angiogenesis needed in endothelial layer repair and regeneration.

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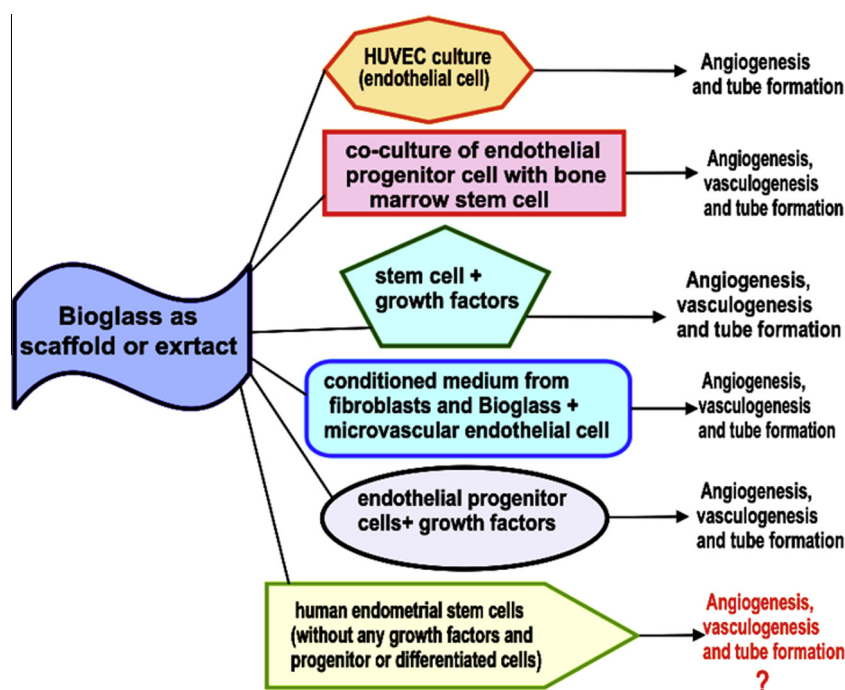
## Introduction

Endothelial layer dysfunction is a sign of beginning of vascular diseases such as coronary artery disease [1], hypertension [2], and vascular complications of diabetes [3], hypercholesterolemia [4], chronic renal failure [5], and atherosclerosis [6]. It is closely attributed to risk factors containing family history, age and ethnicity (cannot be changed), smoking, unhealthy diets, obesity, physical inactivity, diabetes, high cholesterol levels, and hypertension (can be changed or treated) [7–10]. Recent studies showed that stem cells therapy and transplantation of progenitor cells have opened up new promising strategies in treatment of vascular diseases by vascular regeneration and enhancing angiogenesis [11–14]. Stem cells are undifferentiated cells characterized by their potency to proliferate, develop specific committed progenitor cells and their capacity to differentiate tissue specific cells [15]. The use of embryonic stem (ES) cells for clinical trials is high on the political and ethical agenda. The process to obtain bone marrow stem cells is invasive procedure and can cause bleeding, infection and chronic pain, so restricting a broad usage of bone marrow stem cells in cell therapy. Finding a suitable source for stem cell-based therapy that can be applied in the clinic for vascular regeneration and repair is a challenge. Angiogenesis is the new blood vessels formation from proliferation of endothelial cells (differentiated and mature cells) of pre-existing blood vessels [16] but vasculogenesis is the de novo formation of blood vessels using mesodermal stem cells (undifferentiated cells) into the endothelial cells lineage [17]. In vasculogenesis process, the stem cells that are differentiated to form endothelial progenitor cells (EPCs), a typical precursor for mature endothelial cells [18]. A stem cell with more limitation of its differentiation capacity or a unipotent stem cell is called a progenitor and can generally differentiate into a particular type of cell. Two common sources of EPCs exist in the human, one in

the circulating blood and bone marrow and the other residing within the vessels wall [19–21]. In vivo studies showed that EPCs contributed to endothelial cells turnover and so could be used therapeutically to increase vascular function [22]. Vascular regeneration and angiogenesis are influenced by the number of functional EPCs, stimulation of EPCs differentiation, critical concentrations of Calcium(Ca), Copper(Cu), Magnesium(Mg) ions and formed by cytokines and various growth factors including FGFs, EGF, PDGF, VEGFs, and IGF-I [23]. In this hypothesis, we studied angiogenic and tube formation capacities of human endometrial stem cells (uncommon source of EPCs) in the presence of angiogenic agent such as BG, and therapeutic possibilities of human endometrial stem cells therapy with angiogenic materials (as scaffolds) for new blood vessel formation. Thus, the plan currently hypothesized to provide a novel therapeutic strategy and ameliorate endothelial dysfunction may result in decreased mortality and morbidity in vascular injury.

## The hypothesis

In recent years, the therapeutic proficiency of stem and progenitor cells in endothelial vascular disorder is an important and exciting aspect of cardiovascular research. There are stem/progenitor cells within the blood vessel walls and in the circulating blood system (originated from bone marrow) that are capable of differentiating into endothelial cells. Previous studies showed that bioglass (BG) stimulate the secretion of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (angiogenic growth factors) from human fibroblast cells, and conditioned medium from human fibroblast cells significantly enhanced the proliferation of human microvascular endothelial cells (angiogenesis in vascular endothelial cells). This material promotes vasculogenesis and angiogenesis in the presence of endothelial growth factors



**Fig. 1** Schematic illustration of the induced angiogenesis and tube formation hypothesis using bioglass (BG) scaffold or extract.

(exogenous agent), co-culture of progenitor cells and stem cells, and non-stem like cells such as differentiated cells, progenitor cells (Fig. 1). Thus, our hypothesis was that application of human endometrial stem cell (as a new source for increasing the number of EPCs) with BG (as extract or scaffold) may increase number of EPCs, so if lost endothelial cells could be expeditiously substituted by exogenous EPCs that are originated from human endometrial stem cells, enhanced vascular repair and regeneration might be achieved.

## Evaluation of hypothesis

### Endometrial stem cells properties

Human endometrial stem cells (hEnSCs) were demonstrated to have dynamic remodeling roles in uterine cyclic renewal [24] and partake a distinguished contribution in regeneration and repair of endometrium [25]. They were showed to be immuno-privileged in comparison to other stem cell types, isolating hEnSCs a new insight for stem-cell-based therapies and rendering these stem cells as a novel candidate in regenerative medicine. It has been demonstrated that hEnSCs proliferate rapidly in cell culture, as well as have anti-inflammatory characteristics increasing the wound repair with new tissue formation and abatement of fibrosis [26]. Their differentiation potential to mesoderm-derived cell lineages, such as neuron [27], osteoblasts [28], cartilage [29], and hepatocyte [30] were previously shown. As shown in recent studies the hEnSCs are positive for mesenchymal stem cell markers such as CD44, CD90, CD146, and CD105 and are negative for CD34 (endothelial progenitor cell marker) and CD31 (mature endothelial cell marker) [31].

### Angiogenic properties of bioactive glass

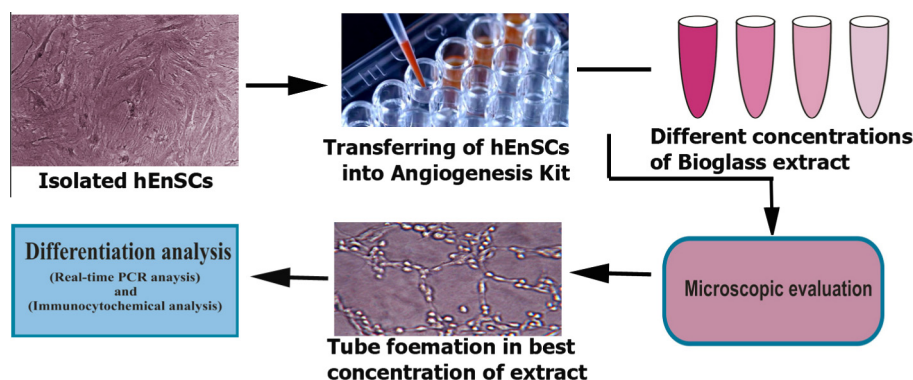
Bioglass (BG), family of bioactive glasses, because of its high bioactivity, biocompatibility, and composition stimulates osteogenesis and angiogenesis in vivo. BG, depending on its application, can be synthesized with various chemicals like  $\text{SiO}_2$ ,  $\text{Na}_2\text{O}$ ,  $\text{CaO}$ , and  $\text{P}_2\text{O}_5$ . These chemical components can be substituted with different functional groups such as  $\text{MgO}$ ,  $\text{K}_2\text{O}$ , and  $\text{CaF}_2$ . Recent studies have shown the capacity of bioactive glass to increase angiogenesis through dissociation

products of BG, which is important to many applications in the wound healing and tissue repair and regeneration. Inducing endothelial differentiation of hEnSCs with biomimetic BG material may improve the number of EPCs, endothelial regeneration and neovascularization due to angiogenesis.

## Description of the study

Hypothetical sketch (Fig. 2) involves different concentration of BG extracts for the assessment of a suitable BG dose for inducing spontaneous angiogenesis in hEnSCs. Our propounded process involves the following steps:

1. The sol-gel prepared BG powder ( $\text{SiO}_2$ ,  $\text{MgO}$ ,  $\text{CaO}$ , and  $\text{P}_2\text{O}_5$ ) was synthesized according as described previously [32]. The solution preparation was described as follows: 13.13 g of tetraethyl orthosilicate (TEOS) was mixed into 30 ml of 0.1 M nitric acid ( $\text{HNO}_3$ ), the mixture was agitated for 45 min at room temperature for the acid hydrolysis of TEOS to begin approximately to completion of reaction. The other reagents were added in progression allowing an hour for each material to entirely react: 0.91 g of triethyl phosphate (TEP), 6.14 g of calcium nitrate tetra-hydrate ( $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ), and 1.28 g of magnesium nitrate hexahydrate ( $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ). The solution was preserved in an isolated Teflon container for 10 days at room temperature to provide the polycondensation and polymerization reaction to occur until the BG gel structure was formed. Then, the gel was heated at 70 and 120 °C for 3 days to remove all the water. The dry powder was heated for 24 h at 600 °C for sintering and nitrate elimination. Energy-dispersive X-ray spectroscopy (XRD) and Fourier transform infrared (FTIR) spectroscopy were performed to assess the atomic elemental analysis and chemical band structure of powder, respectively.
2. Isolation of hEnSCs from 30 years old donor without any urogenital diseases. The patient was apprised about the study and signed the consent forms voluntarily. The sample was maintained in 10 ml of Hanks' balanced salt solution (HBSS) with 1% (v/v) penicillin/streptomycin. The tissue was digested in 0.3% (w/v) collagenase type I at 37 °C for 45 min, neutralized by Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin, and



**Fig. 2** Demonstrative illustration of the stages that require in the human endometrial stem cells (hEnSCs) differentiation into endothelial progenitor cells and mature endothelial cells via the bioglass (BG) extract induction.

centrifuged at 1500 rpm for 5 min. The pellet of cells was resuspended in DMEM containing 10% FBS and 1% penicillin/streptomycin. Cell culture was incubated in 5% (v/v) CO<sub>2</sub> at 37 °C for 10 days. The medium was replaced every 3 days. Flow cytometric test of isolated hEnSCs is performed for hEnSCs markers.

3. Preparation of BG extract. 1 g BG powder was incubated in 5 ml DMEM media for 7 days at 37 °C. After that, supernatant was collected and sterilized with 70 µm filter.
4. Assessment of vasculogenic and angiogenic potentia of BG powder extract on hEnSCs.

The vasculogenesis potentials of BG on hEnSCs were evaluated by using an In Vitro Angiogenesis Assay Kit (Millipore, Cat. No. ECM625). This kit is useful for testing the effects of stimulatory or inhibitory exogenous signals such as growth factors, drugs and toxic agents. The kit includes a solid gel of basement proteins prepared from laminin, collagen type IV, heparan sulfate proteoglycans, entactin, and nidogen without any differential factors such as VEGF. We examined the vasculogenesis, angiogenesis by hEnSCs and HUVEC (as a control) in a 96-well tissue culture plate.  $5 \times 10^4$  hEnSCs/scaffold of the 2nd passage were transferred to each well of kit and incubated for 12 h at 37 °C. Different concentrations of BG extracts were added to each well, incubated for 6 h at 37 °C. Tube formation is inspected under an inverted light microscope at 20× magnification. After that, Real-time PCR analysis is performed to evaluate the expression of a set of EPCs and endothelial cells gens such as FLT1, PECAM, ECAD and vWF markers, and immunocytochemistry procedures are performed for studying expression of CD34 and CD31.

## Discussion

Recently, there has been an increasing attentiveness in the potential of utilizing a cell-based therapy with exogenous angiogenic agents such as VEGF and FGF-2 proteins to attain regenerative angiogenesis, vasculogenesis and treatment of endothelial vascular disorders [33,34]. Previous studies showed that BG (Fig. 1) possess angiogenic potential over a small range of concentrations, promote the release of VEGF and bFGF angiogenic proteins [35]. Endothelial dysfunction in myocardial infarction [36], brain stroke [37], chronic renal failure [5], diabetes [38] and hypertension diseases [39,40] are attributing to decrease number of EPCs and impair activity of the these cells. An in vivo animal model with acute myocardial or ischemic disorders can be useful to investigate the vasculogenic, angiogenic capacity of hEnSCs via the usage of BG in control or recovery model of disease by ameliorating clinical symptoms, evaluating cell markers expression in hEnSCs-derived EPCs and angiogenesis in histological sections (staining for endothelial cell markers such as vWF or other markers) of the defect site of rat heart.

## Conclusion

The current study reveals that hEnSCs culture with BG extract can induce spontaneous vasculogenesis and angiogenesis by enhancing the number of EPCs and endothelial tube formation, so a possible new application of angiogenic agent (BG)

with immuno-privileged, readily available sources of adult stem cells like human endometrial stem cells will promote a good choice for endothelial repair and vascular tissue engineering.

### Overview box:

#### **First question: What do we already know about the subject?**

Endothelial dysfunction has been betokened as a key phenomenon in the pathogenesis of ischemic diseases and coronary vasoconstriction. The application of hEnSCs with scaffolds and growth factors can be extensively advantageous based on rising proof in the literature about the multiple differentiation potency of this stem cell. Also, it has been shown in animal models that bioglass delivery to ischemic tissues greatly promote compensative angiogenesis.

#### **Second question: What does your proposed theory add to current knowledge available and what benefits does it have?**

Our hypothesis suggests that using human endometrial stem cells (as the new source for increasing the number of endothelial progenitor cells in endothelial dysfunction diseases) with bioglass (angiogenic agent), and also assessing its relating cellular and molecular processes could be a promising strategy for treatment of ischemic and endothelial disorders.

#### **Third question: Among numerous available studies, what special further study is proposed for testing the idea?**

We suggest that human endometrial stem cells with bioglass could propose a suitable new insight for increasing hEnSCs-derived EPCs in vascular tissue engineering. We induce myocardial infarction in vivo model with coronary ligation in C57BL/6 mice and insertion bioglass-based scaffold (for controlled release of bioglass ions) seeded with human endometrial stem cells in mouse model, to examine the effects of BG material with human endometrial stem cells in clinical symptom improvement and assessment of circulating endothelial progenitor cells number compared to control models without human endometrial stem cells.

## Conflict of interest

The authors declare that they do not have any conflict of interest.

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